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19OCT98 E398151-1 D02000
P01/7700 0.00 - 9822682.2

2. Patent application number *(The Patent Office will fill in this part)* — 16 OCT 1998 **9822682.2**

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Gemini Research Ltd
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Patents ADP number *(if you know it)* — 7434614001

If the applicant is a corporate body, give the country/state of its incorporation — GB

4. Title of the invention

DIAGNOSTIC METHOD AND APPARATUS BASED ON POLYMORPHISM IN A TGF-ß GENE

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP number *(if you know it)* — 1081001

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Signature: Matthew Squire   Date: 16 October 1998

12. Name and daytime telephone number of person to contact in the United Kingdom: George W Schlich   0171 830 0000

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DIAGNOSTIC METHOD AND APPARATUS BASED ON POLYMORPHISM IN A TGF-ß GENE

This invention relates to diagnostic method and apparatus based upon polymorphism of a TGF-ß Gene. More specifically, this invention relates to a method for diagnosis of pre-disposition to certain disease states, particularly osteoporosis, by screening for the presence of this polymorphism. The invention also relates to apparatus for screening for the polymorphism. The invention further relates to TGF-ß genes containing a polymorphism and to a probe therefor.

Hormone replacement therapy is an established treatment for osteoporosis and has proved successful in halting further decline in bone density that is characteristic in women suffering from this disease. Hormone replacement therapy is generally not, however, able to bring about a reversal of osteoporosis, that is to say it is not capable of inducing an increase in the bone density of sufferers. The same criticism is made of other known treatments for osteoporosis.

It would, accordingly, be of particular advantage to be able to identify with increased accuracy those individuals having a predisposition or increased susceptibility to osteoporosis. Suitable therapy could then be put into place before the effects of osteoporosis set in.

Genetic factors play an important role in determining bone mineral density (BMD) in later life, with the genetic influence mediated through effects on both peak mass and on age- and menopause- related bone loss. At the menopause there is an increase in the production and activity of various cytokines and growth factors within the bone microenvironment.

Bone mineral density (BMD) in later life is a strong predictor of subsequent osteoporotic fracture and is determined by both the peak value achieved during skeletal growth and by age- and menopause- related bone loss. Family and twin studies suggest a strong genetic component to the determination of peak bone

mass, with 50-85% of the population variance in BMD being attributable to genetic factors. Twin studies in postmenopausal and elderly women also support a persistent and significant genetic influence on bone mass in later life. This may represent either a strong residual effect from the genetic contribution to peak bone mass or an independent genetic effect on the regulation of bone loss. Indirect assessment of bone turnover through biochemical markers suggests a genetic regulation of bone metabolism that may translate into differing effects on bone loss although to date only two twin studies have directly attempted to explore the genetic contribution to age- and menopause- related bone loss with conflicting and uncertain results.

Osteoporosis is a complex disease that is likely to have a polygenic aetiology, and candidate gene analysis has demonstrated that polymorphisms of the vitamin D receptor (VDR) locus the oestrogen receptor (ER) locus and the type I collagen alpha 1 (COL1A1) locus are all potential genetic markers for bone mass and bone loss.

The search for further genetic markers for use in diagnosis of disease, including diagnosis of osteoporosis and predisposition thereto, nevertheless continues.

It is an object of this invention to provide method and apparatus for detecting individuals having a predisposition or susceptibility to osteoporosis. It is a further object of the invention to identify individuals having such a predisposition or susceptibility by identifying those individuals on the basis of genotype. It is another object of the invention to provide a therapy for those individuals. Still further objects of the invention are to provide an isolated gene comprising a polymorphism indicative of predisposition to osteoporosis and probe therefor.

Accordingly, a first aspect of the invention provides a method of diagnosis comprising determining genotype of an intron of a TGF-ß gene.

The method of the invention typically comprises determining whether an individual

is homozygous or heterozygous for a the gene and a particular polymorphism thereof. The method is conveniently used to screen for an individual at risk of a condition or disease correlated with a polymorphism of this gene.

The method of the invention determines whether the individual being tested has a TGF-ß gene which is identical with the published sequence or whether that individual has a gene which differs from the published sequence, i.e. is a polymorphism of the published sequence. In carrying out the invention, an individual's TGF-ß gene genotype is generally determined by analysis of a section of the gene, rather than by analysis of the entire gene. If the sequence of that section is found to be the same as the corresponding section in the wild type sequence, then that individual is classified as having the wild type gene.

In use of an embodiment of the invention to be described below in further detail, an individual is screened to determine whether he or she possess a TGF-ß gene which is the published sequence or is a polymorphism thereof in which there is a polymorphism in one or more of its introns. In this specific embodiment, the presence of a homozygous polymorphism in an intron of the gene correlates with a predisposition to osteoporosis.

Screening is carried out, for example, using PCR primers adapted to amplify a portion of gene in the region of and including the site of the polymorphism It is preferred that the PCR primers are selected so as to amplify a region of the gene that surrounds the region and includes at least six nucleotides on either side. PCR techniques are well known in the art and it would be within the ambit of a person of ordinary skill in this art to identify primers for amplifying a suitable section of the gene. PCR techniques are described for example in EP-A-0200362 and EP-A-0201184.

A second aspect of the invention provides diagnostic means comprising PCR primers adapted to amplify a region of a TGF-ß gene, preferably a DNA segment comprising intron 5.

Suitable primers are recited in the example below. The invention further provides a diagnostic kit comprising diagnostic means according to the second aspect of the invention, optionally within a container.

A third aspect of the invention provides DNA probes capable of distinguishing between a wild type gene, to which the probe does not bind, and a polymorphism thereof, such as one containing a polymorphism in intron 5 to which the probe does bind.

The invention is of advantage in that by screening for the presence of the polymorphism it is possible to identify individuals likely to have a genetic predisposition to this disease.

Accordingly, a fourth aspect of the invention provides a method of therapy comprising screening an individual for a predisposition to osteoporosis and, if a genetic predisposition is identified, treating that individual to delay or reduce or prevent the osteoporosis.

A suitable treatment to prevent or reduce or delay osteoporosis is hormone replacement therapy. The use of this therapy is well known in the art. According to the invention, hormone replacement therapy can thus be commenced in individuals likely to have a predisposition to osteoporosis but in whom osteoporosis has not yet begun to any significant extent.

It is believed that the use of hormone replacement therapy carries with it a concomitant increased risk of breast cancer. The invention offers the advantage that the increased risk of breast cancer associated with hormone replacement therapy can be accepted only by those women who are known to have a likelihood of predisposition to osteoporosis. In an embodiment of this aspect of the invention, the predisposition of an individual to osteoporosis is assessed by determining whether that individual is homozygous for the wild type TGF-ß gene, is heterozygous for the wild type and the, or is homozygous for the polymorphism -

indicating risk of predisposition to osteoporosis.

According to the invention, an individual who is homozygous for the risk polymorphism is classified as being at highest risk.

Another suitable treatment is use of bisphosphonates. Two specific treatments involve using xanthine oxidase inhibitors or substituted benzodiazepines and are described in US-A-5436258 and US-A-5441964, the contents of which are incorporated herein by reference. Still further treatments will be known to a person of skill in the art. Potential treatments are described, for example, in JP-A-09030977, WO-A-97/06254, JP-A-09025293, WO-A-97/04799, WO-A-97/03060 and JP-A-09012592, the contents of which are incorporated herein by reference. Currently authorised treatments for osteoporosis include the use of oestrogens, with and without progestogen, the use of selective oestrogen receptor modulators, the use of anabolic steroids such as nandrolone, the use of the bisphosphonates alendronate and disodium etidronate, the use of salcatonin and administration of calcium supplements.

In pharmaceutical treatment of osteoporosis, all routes of administration are suitable and include but are not limited to oral, injection intravenously, intraperitoneally, intramuscularly and subcutaneously, intranasal and topical administration. Typical dosages and durations of treatment are as described in clinician's textbooks such as British National Formulary, incorporated herein by reference.

Currently, none of the osteoporosis medications that have been approved by the Food and Drug Administration (FDA) for postmenopausal women have been approved for men.

Testosterone replacement therapy may be prescribed for a man with a low testosterone level.

Calcitonin is a medication that slows or stops bone loss and may relieve the pain of fractures in some patients. Calcitonin is approved by the FDA for the treatment of osteoporosis in postmenopausal women. While its effect in men has not been studied, evidence suggests that it may work the same in men as in women. Calcitonin is available as an injection and as a nasal spray. Its use is described in US-A-5440012, incorporated herein by reference.

Bisphosphonates are a class of drugs that have been shown to help preserve and increase bone density by slowing or stopping bone loss. The FDA has approved the bisphosphonate known as alendronate for the treatment of postmenopausal osteoporosis in women; it is currently being studied for treatment of osteoporosis in men. There are other bisphosphonates under development - and in fact etidronate has been approved, though only outside the USA.

Sodium fluoride has recently been recommended for approval by an FDA committee. Parathyroid hormone, calcitriol, and others are investigational drugs. It will be some time before research findings are available on these preparations.

Decrease in bone mineral density can also be slowed by taken calcium supplements, and some suggested levels are 1,000 mg of calcium a day for women on oestrogen replacement therapy and 1,500 mg of calcium daily for women not receiving oestrogen therapy.

Thus, a range of treatments for those suffering or predisposed to osteoporosis are known and all are believed suitable for use in combination with its diagnosis according to the present invention.

Optionally, the assessment of an individual's risk factor is calculated by reference both to the presence of a TGF-ß gene polymorphism and also to other known genetic or physiological or dietary or other indications. The invention in this way provides further information on which measurement of an individual's risk can be based.

A still further aspect of the invention is that a further polymorphism may be so correlated with presence of the TGF-ß polymorphism of the invention that the two polymorphisms are in linkage disequilibrium. Thus, diagnosis of disease by determining genotype of the further polymorphism may lead to a similarly reliable diagnosis of osteoporosis or predisposition thereto.

The invention thus also provides a method of identifying, and optionally treating, an individual predisposed or susceptible to osteoporosis, said method comprising determining genotype of a first gene in said individual, wherein genotype of said first gene is correlated with genotype of a TGF-ß gene in said individual.

The invention yet further provides a method of identifying a further polymorphism correlated with predisposition to osteoporosis, comprising identifying in a cohort of individuals with polymorphisms of the TGF-ß gene a further polymorphism and determining whether that further polymorphism is correlated with polymorphism of the TGF-ß gene. The further polymorphism may preferably be on the same gene, but can also be on any gene.

The invention further again provides a method of predicting response to osteoporosis therapy, comprising diagnosing genotype of a TGF-ß gene, in accordance with the first aspect of the invention.

This latter aspect of the invention thus enables informed choice of therapy, including choice of type of therapy and choice of amount or strength of therapeutic agent, to be made for a given individual predisposed to osteoporosis. Moreover, for a given individual already suffering from osteoporosis, the invention enables an assessment of whether the currently prescribed therapy is likely to be effective in treating the disease or if an alternative therapy regime will be more successful. In a specific embodiment of the invention, diagnosis of a risk polymorphism in a TGF-ß gene indicates that hormone replacement therapy, or an equivalent, is likely to be effective. More specifically, possessing two copies of the risk polymorphism indicates an increased level of therapy is likely to be appropriate.

Bone mineral density (BMD) in later life is a major determinant of osteoporotic fracture risk and has been shown to be under strong genetic influence. Segregation analysis within families and data from twin studies has suggested that this genetic effect on BMD is probably mediated by a number of genes each having small individual effects. Transforming growth factor ß (TGF-ß) is an important regulatory cytokine and is found in high concentrations in the bone matrix. TGF-ß is therefore a plausible candidate for the genetic regulation of BMD. In total 911 DZ pairs and 386 MZ pairs (age range 18-76 years) were studied, with measurements of BMD using DXA and calcaneal ultrasound. In accordance with the present invention, a novel T/C polymorphism was identified in intron 5 with an allele frequency of 0.25 within the DZ subjects. Comparison of the variance in femoral neck BMD between the MZ and DZ twins showed a heritability of 62% at this site. BMD at the femoral neck was 5% lower in subjects homozygous for the presence of the TGF-ß polymorphism when compared to the other two genotype groups. No effect was seen at the lumbar spine, ultradistal radius, or with ultrasound measurements. Results were unaffected after adjustment for potential confounders. Linkage analysis within the DZ twin pairs confirmed the significance of this polymorphism on hip BMD.

Osteoporosis is a common age-related condition characterised by reduced bone mineral density (BMD), deterioration in skeletal microarchitecture and an increased risk of fragility fracture. One in three Caucasian women will experience an osteoporotic fracture during their lifetime, and these fractures are a major cause of morbidity and mortality leading to a massive health care cost, estimated at $14 billion per year in the USA. BMD is the strongest predictor of fracture (3) and a large number of twin and family studies have suggested a strong genetic influence on this trait, with up to 85% of the population variance in BMD being attributable to genetic factors (4,5,6).

Normal skeletal morphogenesis is dependent on a complex interaction between osteoblasts, osteoclasts, and local growth factors. During growth and development the processes of osteoblastic bone formation and osteoclastic bone

resorption appear coupled, thereby maintaining skeletal integrity and preserving bone mass and shape. Non-invasive measures of bone turnover have shown that after the menopause there is an increase in bone resorption, leading to a loss of bone and subsequent development of osteoporosis. Several studies have also shown that in women with osteoporotic fracture the remodelling balance is more negative when compared to age-matched women with no fracture history. It is believed that this postmenopausal increase in bone resorption in the oestrogen deficient state is mediated in part by cytokines (interleukins 1 and 6, tumour necrois factor () and various growth factors (transforming growth factor (, insulin like growth factors). Twin studies have also suggested that there is a genetic influence on the general process of bone turnover in pre- and post-menopausal women, with higher correlations for biochemical markers of both bone formation and resorption seen in identical compared to non-identical twins (13,14).

Transforming growth factor ß (TGF-ß) is synthesised by osteoblasts and osteoclasts in vivo and has three isoforms. High concentrations of all these isoforms can be extracted from the mineralised bone matrix, and although TGF-ß is found in a variety of tissues, the concentration of TGF-ß appears to be highest in the bone matrix. Osteoblasts produce TGF-ß largely as a matrix-bound latent complex composed of 390 amino acids. It is only released during bone resorption, with subsequent activation in the acidic environment below the ruffled border of the resorbing osteoclast (17,18). This suggests that TGF-ß may play a central regulatory role in the coupling that exists between bone formation and resorption. Active TGF-ß is formed of two identical disulphide-linked polypeptide chains consisting of the 112 amino acids from the C-terminal part of the precursor protein. The TGF-ß gene maps to chromosome 19q13 and contains 7 exons. The active component of TGF-ß is encoded by part of exon 5, exon 6, and part of exon 7. The TGF-ß gene may therefore be an important candidate gene for the development of osteoporosis, with variation at this locus being associated with differences in BMD.

The present invention is now illustrated by way of the following example.

METHODS

Subjects

The subjects studied were Caucasian, female monozygous (MZ) and dizygous (DZ) twins (age range 18-76 years) recruited after national media campaigns. All subjects were healthy and did not suffer from diseases specifically affecting bone, and were broadly representative of the normal United Kingdom population as previously described. Twins completed a nurse-administered questionnaire detailing medical, obstetric and gynaecological histories, full drug histories, dietary calcium assessment, exercise levels, smoking status, and alcohol intake. Twin zygosity was determined by questionnaire and in doubtful cases this was confirmed with multiplex DNA fingerprinting.

Measurements

BMD was measured at the lumbar spine (L1-4), non-dominant hip (femoral neck, total hip), and non-dominant ultradistal radius using DXA on a Hologic QDR-2000. Reproducibility as assessed by the coefficient of variation (CV%) from duplicate measures in healthy volunteers was between 0.8% and 1.6% at the skeletal sites measured. Subjects were classified as having osteoporosis according to the World Health Organisation diagnostic criteria if their BMD measurement was 2.5 standard deviations (SD) below the mean peak young adult value (i.e. T –score < -2.5).

Calcaneal ultrasound was measured using a McHue Cuba Clinical scanner. This produced two output variables: broadband ultrasound attenuation (BUA) and velocity of sound (VOS). Reproducibility as assessed by the CV% in duplicate measures on 30 subjects was 2.5% (BUA) and 0.44% (VOS).

Polymorphism identification

DNA was prepared for each subject from peripheral blood leucocytes using a

standard phenol extraction method. Common single nucleotide polymorphisms (SNPs) in the TGF-ß gene were detected by sequence analysis of 24 unrelated dizygotic (DZ) individuals and comparison made with the published sequence (Accession no. Y00112) (20,21). This strategy was chosen to identify polymorphisms with an allele frequency of at least 0.1 within the study group. Oligonucleotide primer pairs were designed to cover the TGF-ß1 coding regions, and the promoter and 5 -untranslated region up to position 1363. Following amplification by the polymerase chain reaction (PCR) the products were purified with the Advanced Genetic Technologies Corp 96 well PCR purification system and sequenced using PE Applied Biosystems dRhodamine Terminator cycle sequencing kit. The sequencing reactions were analyzed on an ABI 377 DNA sequencer.

Restriction enzyme digests

Polymorphism screening within the DZ group was performed using PCR-restriction fragment length polymorphism (RFLP) based methods with restriction enzyme digest. Two PCR-RFLP assays were used in this study:

BstUI PCR-RFLP analysis of the intron 5 SNP

PCR amplification was performed using the following oligonucleotide primers: 5'-ATGGTGGTAGCCCCTCCCT-3' and 5'-GCATCTCGTAGCCCGGTGG-3'. Reactions were performed in 25(l with the following composition: 0.3uM primers, 0.2mM dNTPs, 1mM MgCL2, 1X Taqgold buffer, 1.25 units Taqgold (P.E. Applied Biosystems) and 50ng genomic DNA. Thermocycling was performed on a MJ Research DNA Engine Tetrad PTC-225 thermal cycler using the following conditions: 95oC for 14 minutes, 35 cycles of 94oC for 15 seconds, 60oC for 15 seconds, 72oC for 30 seconds, followed by final extension of 72oC for 10 minutes. The 230 bp PCR product was digested with 3 units of BstUI (New England Biolabs) at 60oC for 2 hours producing polymorphic fragments of 202 and 28 bp. Products were analysed by agarose gel electrophoresis with size determination after transillumination under ultra-violet light. Alleles were coded as

A1 = presence of restriction site, and A0 = absence of site.

Stul PCR-RFLP analysis of the codon 255 SNP.

PCR was performed as described above execpt for the following modifications: the oligonucleotide primers used were 5'-ACTGCTCCTGTGACAGCAG-3' and 5'-ATCCAGGCTACAAGGCTCAC-3' and the annealing temperature was reduced from from 60(C to 55(C. Digestion of the 354 bp PCR produc was performed with 3 units of Stul (New England Biolabs) at 37(C for 2 hours.

Statistical analysis

Differences in the mean value in each variable for the three TGF-ß genotypes were tested using generalised estimating equations (GEE).22 This method takes into account the dependence of measurements within twin pairs in estimating the significance of the differences. The GEE models were extended to include potential confounding variables (age, menopausal status). Significant GEE results were followed up using a quantitative genetic modelling approach in which additional phenotypic data on MZ twins were used. A model was specified which provided estimates for the genetic and environmental variance components and estimates for the means of each possible genotype (A0A0, A0A1 and A1A1). This enabled us to estimate the percentage of the genetic variation in the phenotype that could be attributed to the TGF-ß polymorphism. Model fitting was performed using Mx. Parameters were estimated by normal-theory maximum-likelihood, where the model were fitted to the raw data. Linkage analysis within the DZ twins was also analysed using the MAPMAKER/SIBS programme (version 1.0). This calculates the maximum likelihood sharing probabilities for each DZ pair and as parental information was not available this utilised estimated allele frequencies from within the total DZ group. Evidence for linkage of the polymorphism to the trait was taken as a nominal lod score (1.00 or a nominal P value (0.05.

Results

Sequence analysis of 24 unrelated individuals identified two novel polymorphisms in the TGF-ß gene in addition to six previously identified polymorphisms within the 5′ and coding regions. The two novel polymorphisms included a C(A substitution at codon 255 and a T(C substitution in intron 5, 20bp upstream of exon 6. The first substitution in codon 255 is a synonomous codon change (CCG to AGG) and has no affect on the amino acid sequence at this point (arginine). Subsequent testing of this polymorphism using a StuI PCR-RFLP showed, however, that this allelic variant was very rare as it was only identified in one individual out of 400 subjects tested. The polymorphic T(C substitution in intron 5 introduces a BstUI site (recognition sequence 5′-CG(CG-3′), and subsequent screening for this within the whole DZ cohort using the BstUI PCR-RFLP showed the allele frequencies to be 0.75 and 0.25, with the genotype distributions being in Hardy Weinberg equilibrium.

In total, data was available on 911 DZ and 386 MZ twin pairs. Intron 5 genotype results were available on 1664 of the DZ subjects. Reasons for absence of genetic results included inadequate DNA extraction or failure of the PCR-RFLP assay. No significant differences were observed, however, between subjects with and without TGF-ß genotype results. Characteristics of the study population are shown in Table 1. In comparison with the DZ twins, the MZ twins were on average slightly older by 2 years and had a higher percentage of women who were postmenopausal. Within the DZ group there were no differences in age, height, weight, smoking status or hormone replacement (HRT) use between the three TGF-ß intron 5 genotype groups. There were however, small differences in the proportion of postmenopausal women (Table 1).

The TGF-ß intron 5 genotype A1A1 was associated with hip BMD when compared to the other 2 genotypes, with a 5% reduction in femoral neck BMD and a 3.8% reduction in total hip BMD (Table 2). These findings were unaltered after adjustment for the small difference observed in menopausal status. These results

at the hip, suggest a recessive pattern of risk associated with carriage of the rarer A1 TGF-ß intron 5 allele. No genotypic association was seen at the lumbar spine, ultradistal radius or in the calcaneal ultrasound parameters of BUA and VOS. The prevalence of clinically defined osteoporosis (T-score <-2.5) at the femoral neck in the genotype group A1A1 was 18% compared to 10% in the A0A0 and A0A1 groups. This demonstrates a 70% increased risk for a subject with the A1A1 genotype having femoral neck osteoporosis when compared to the other 2 genotypes, with an odds ratio (95% confidence interval) of 1.71 (0.95, 3.07), P = 0.07.

Having demonstrated an association between TGF-ß intron 5 genotype and hip BMD, twin modelling and variance component analysis was utilised to estimate the proportion of the genetic variance explained by this polymorphism. This analysis confirmed previous studies showing high heritabilities for BMD and ultrasound parameters at the various skeletal sites. At the femoral neck the estimated heritability was 0.62, with the proportion of the population genetic variance explained by the intron 5 TGF-ß polymorphism at this site being 0.60%.

The results of the single point linkage analysis are shown in Table 3. These demonstrate linkage between the intron 5 polymorphism and femoral neck BMD. For the measurement of BUA the nominal P value approaches 0.05 although the lod score does not exceed 1.00.

Thus, in accordance with the present invention, a novel T(C polymorphism in a TGF-ß intron, specifically intron 5 in an embodiemtn of the invention, of the human TGF-ß gene. Our data demonstrate both association and linkage between this polymorphism and hip BMD in a large group of unselected, normal female twins. The polymorphic C allele was present at a relatively high allelic frequency in the female population (0.25), and there appeared to be a recessive pattern of risk associated with this allelic variant. Hip BMD was 3-5% lower in women who were homozygous for the carriage of the polymorphic allele, when compared to women who were either heterozygous or homozygous for the commoner allele. Women

who were homozygous for the polymorphism also had a 70% increased risk of having osteoporosis at the femoral neck in comparison to the other two genotypes.

These cross-sectional data indicate that subjects who are homozygous for an intronic polymorphism of the TGF-ß gene have reduced BMD at the femoral neck and total hip. The observation that these findings were confined to the hip (and predominantly at the femoral neck), rather than being seen at the spine and distal radius suggest a site-specific association. The absence of any genotype association with bone ultrasound measures also suggests that the TGF-ß intron 5 polymorphism has a predominant effect on BMD rather than on bone quality or structure. In the future, case-control and longitudinal studies will be required to determine whether this genetic variant has also any effect on fracture risk. It will also be important to determine if this genetic effect is mediated through a predominant action on peak BMD or whether there is an influence on rates of either age- or menopause-related bone loss. Although the proportion of population variance in BMD attributable to this polmorphism appears small, this reflects the recessive risk associated with this locus as only approximately 6% of the population would be expected to be of the genotype A1A1. Our data indicate, however that these subjects have a 70% increased risk of having osteoporosis and are therefore at increased risk of fracture. TGF-ß genotype could therefore identify an at risk sub-group of women who could benefit from targeted intervention. This is also illustrated by recent findings from a large population study examining the relationship between a polymorphic variant in the promoter region of the type I collagen 1(gene and osteoporosis. In this study the polymorphic allele was associated with a 2-fold increased risk of fracture despite only explaining 0.3 to 0.4% of the population variance in BMD.

There is currently a large amount of data suggesting that TGF-ß may play a central role in the regulation of BMD and bone turnover. In vivo studies have shown that local injection of TGF-ß under the periosteum stimulates cartilage and bone formation (28) and that systemic injection of TGF-ß2 also leads to a generalised increase in osteoblastic activity. In vitro, TGF-ß induces extracellular matrix

secretion by osteoblasts, inhibits matrix mineralisation, and modulates osteoprogenitor cell proliferation. The rate of bone formation is altered in the TGF-ß knockout mouse (30) and administration of TGF-ß corrects the bone density deficiency in elderly mice with osteoporosis. Oestrogen action on bone also appears to be mediated via effects on TGF-ß and induction of osteoclast apoptosis. Allelic variation at the TGF-ß locus may therefore be important in determining the therapeutic response of the oestrogen and selective oestrogen receptor modulators on bone and other tissues. A recent study has also identified a further novel polymorphism in intron 4 of the TGF-ß gene (a C deletion 8 bp upstream of exon 5). This polymorphism was rare, being present in only 10/161 osteoporotic patients with spinal fracture (allele frequency 0.03) and 2/131 controls (allele frequency 0.008). The distribution of this polymorphims differed significantly between the patients and the controls, although there was no significant association between the polymorphism and BMD within these groups as a whole. A sub-group analysis in the osteoporotic patients showed, however, that there was a relationship between lower spinal BMD and carriage of the polymorphic allele in those patients with both fracture and low BMD (defined as Z-score < -1). No relationship was seen at the hip in either the total groups or after sub-group analysis.

The functional significance of our findings on TGF-ß activity is uncertain, although as the polymorphism is 20 bp upstream of exon 6 it could have some influence on the active component of the TGF-ß protein. The lack of coding sequence variation identified in this study and others suggests that the amino acid sequence of the active form of TGF-ß is highly conserved with strong selective pressures against variant proteins. It has been reported that up to 15% of human diseases are caused by point-sequence variation in splice regions resulting in either exon skipping or cryptic splicing (34) and although the intron 5 polymorphism is not located in a slice donor or acceptor site it could affect a branch point. The intronic polymorphic sequence change may also affect messenger RNA stability and further studies will be required to examine these possibilities. If this polymorphism is not functional, then these findings may suggest that the TGF-ß polymorphism is

actually in linkage disequilibrium with a novel disease locus mapping to this chromosomal region. The finding of a positive linkage result at this locus with hip BMD would also implicate this chromosomal region and multipoint linkage analysis will be required to refine the location of the putative disease locus.

The invention thus provides method and apparatus for diagnosis of osteoporosis or predisposition thereto.

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Table 1

Mean (±SD) characteristics of female twin subjects

Variable	MZ total (n=724)	DZ total (n=1758)	DZ subjects according to TGF-β1 intron 5 genotype		
			A0A0 (n = 848)	A0A1 (n = 609)	A1A1 (n = 90)
Age (yrs)	50.1 (13.4)	47.5 (11.3)	47.6 (11.0)	47.5 (11.9)	49.1 (9.6)
Height (cm)	162 (6)	163 (6)	162 (6)	163 (6)	162 (6)
Weight (kg)	63.9 (10.7)	65.9 (12.3)	66.0 (12.1)	65.4 (12.1)	65.4 (12.7)
Subjects postmenopausal (%)	67	54	51	56	62
Subjects ever smoking (%)	44	48	45	49	51
Subjects ever use of HRT (%)	30	30	28	31	31

Table 2

Mean (±SD) BMD at lumbar spine, hip and forearm, and calcaneal ultrasound measurements in DZ subjects according to their TGF-β1 intron 5 genotype

Variable	TGF-β1 Intron 5 Genotype		
	A0A0 (n = 848)	A0A1 (n = 609)	A1A1 (n = 90)
Lumbar spine BMD (g/cm^2)	0.998 (0.147)	1.011 (0.149)	1.005 (0.146)
Femoral neck BMD (g/cm^2)	0.812 (0.131)	0.810 (0.130)	0.770 (0.112)*
Total hip BMD (g/cm^2)	0.924 (0.132)	0.925 (0.131)	0.889 (0.117)‡
Ultradistal radius BMD (g/cm^2)	0.455 (0.068)	0.458 (0.069)	0.451 (0.068)
VOS (m/sec)	1659 (52)	1660 (52)	1661 (51)
BUA (dB/MHz/cm)	78 (19)	78 (19)	76 (17)

* A1A1 vs A0A0, P = 0.005; A1A1 vs A0A1, P = 0.04

‡ A1A1 vs A0A1, P = 0.05

Table 3

Single point lod scores for TGF-β1 intron 5 genotype and BMD/Ultrasound measures in DZ twin pairs.

Variable	TGF-β1 Intron 5	
	Lod score	Nominal P-value
Lumbar spine BMD (g/cm^2)	0.365	0.195
Femoral neck BMD (g/cm^2)	1.091	0.025
Total hip BMD (g/cm^2)	0.479	0.138
Ultradistal radius BMD (g/cm^2)	0.570	0.105
VOS (m/sec)	0.159	0.392
BUA (dB/MHz/cm)	0.785	0.057

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